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Salivary antigens of the cat flea, Ctenocephalides felis felis.
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Thank you,
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# Presence of Calreticulin in Vector Fleas (Siphonaptera)

DEBORAH C. JAWORSKI,[1,2] JAMES A. HIGGINS,[1] SUZANA RADULOVIC,[1] JEFFERSON A. VAUGHAN,[3] AND ABDU F. AZAD[1]

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**ABSTRACT** Calreticulin has been defined in the cat flea, *Ctenophalides felis* (Bouché), and oriental rat flea, *Xenopsylla cheopis* (Rothschild). Calreticulin, a major endoplasmic reticulum protein, was previously identified as a component of ixodid tick saliva. Using a riboprobe generated from the tick calreticulin complementary DNA (cDNA), we distinguished 2 transcripts for calreticulin in cat fleas by Northern blot analysis. Increased expression of calreticulin was not evident in fed versus unfed adult fleas. We were able to amplify a calreticulin flea product from fed female messenger RNA (mRNA) using primers designed from the tick calreticulin gene. One of these products hybridized to the tick riboprobe. Localization of specific antibody to cat flea tissues showed calreticulin in the midgut with no detection in the salivary glands. We also observed specific labeling of calreticulin with antibody in the ovaries of fed females. Several cat flea polypeptides appear to crossreact with anticalreticulin antibody in Western blots. We did not detect a calreticulin using antibody to the tick-secreted protein in cat flea salivary glands. This antibody did recognize a protein in the rat flea salivary glands. Our results show that fleas have calreticulin and, possibly, several isoforms. It appears that the salivary glands of the cat and oriental rat flea differ in detectable levels of calreticulin. The specific antibody labeling of the ovaries is interesting and remains to be understood. Calreticulin's appearance in the midgut suggests a possible source of calreticulin as a flea secretion. Further studies are in progress to complete the sequencing of the flea polymerase chain reaction (PCR) product to compare to tick-secreted calreticulin. Comparisons to other blood-feeding arthropods at the protein and gene level are also being done. We hope to define further the expression of calreticulin in fleas, and in general, blood-feeding arthropods, with respect to its role in feeding and pathogen transmission.

**KEY WORDS** calreticulin, Siphonaptera, flea, blood feeding, arthropod, antithrombosis

DESPITE THE IMPORTANCE of fleas as significant pests of household pets, and their role as principal vectors of bubonic plague and murine typhus, the published data on molecular aspects of flea biology are scant. The alpha subunit of the cat flea sodium pump has been cloned (Reeves and Yamanaka 1993) and some of messenger RNA (mRNA) encoding cat flea, *Ctenophalides felis* (Bouché) allergens have been investigated (Greene et al. 1993). Here we show the results of molecular studies to investigate the flea feeding machinery and, in particular, identify molecular components (i.e., calreticulin) involved in the intermittent feeding behavior. Calreticulin, a multifunctional, calcium-binding protein (see Michalak et al. 1992), was recently identified as a major component of ixodid tick saliva (Jaworski et al. 1995). In ticks, calreticulin may have a role in blood feeding through ADP-degrading enzymes (i.e., apyrase) or the nitric oxide pathway. Calreticulin has antithrombotic activity, which appears to be actuated by the nitric oxide pathway (Kuwabara et al. 1995). Calreticulin as an arthropod secretion could also serve to increase nitric oxide in the feeding lesion leading to increased vascular permeability. This suggestion seems feasible since Ribeiro et al. (1993) demonstrated the presence of nitric oxide in the saliva of the blood-sucking bug *Rhodnius prolixus* (Stål). We are interested in determining whether *C. felis* make and secrete calreticulin. Apyrase has been implicated in flea blood feeding and levels of this enzyme have been determined for the oriental rat flea, *Xenopsylla cheopis* (Rothschild), salivary glands (Ribeiro et al. 1990). Using the tick calreticulin complementary DNA (cDNA), we made a riboprobe to study the expression of calreticulin in the cat flea. Also, we amplified a flea cDNA for calreticulin using calreticulin primers. Additionally, we analyzed cat flea proteins with antibodies to rabbit skeletal muscle calreticulin and to tick-secreted calreticulin. Finally, we compared salivary gland antigens of *C. felix* with *X. cheopis* by immunoblots.

## Materials and Methods

**Fleas.** Adult cat fleas were obtained from Fleadata (Freeville, NY) and maintained at 4°C until

[1] University of Maryland at Baltimore, School of Medicine, Department of Microbiology & Immunology, 655 West Baltimore Street, Baltimore, MD 21201.
[2] Current address: University of California, Irvine, Molecular Biology & Biochemistry, BioSci II, Room 3025, Irvine, CA 92717.
[3] Division of Applied Research, U.S. Army Medical Research, Institute of Infectious Diseases, Ft. Detrick, Frederick, MD 21702.

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used within 24 h after arrival. Unfed adults, fed adults 1 and 14 d after feeding, and eggs were used in experiments to follow. Rat fleas were obtained from the Rocky Mountain Laboratory (Tom Schwan, USDA–ARS, Rocky Mountain Laboratory, Hamilton, MT). Additionally, a cat flea cell primary culture was derived from the eggs of fed females 14 d after emergence. The cell line was propagated in Mitsuhashi and Maramorosch insect media (Sigma, St. Louis, MO) supplemented with 10% Fetal Bovine Serum (Biofluids, Rockville, MD).

**Isolation of RNA.** RNA was isolated from fleas using Trizol Reagent (contains phenol and guanidine thiocyanate; Gibco-BRL, Gaithersburg, MD). Fleas were homogenized directly in 500 μl of Trizol using Kontes tubes and matched pestles (Vineland, NJ). After homogenization, another 500 μl of Trizol was added and samples were stored at room temperature for 5 min. Next, 0.2 ml of chloroform was added, shaken vigorously for 15 s, and stored at room temperature for 15 min. Samples were centrifuged at 12,000 × *g* for 15 min at 4°C. The aqueous upper phase was removed to a new tube and the RNA was extracted with 0.5 ml of isopropanol. Samples were incubated at room temperature for 10 min and centrifuged for 10 min at 4°C. Isopropanol was decanted from the tube and RNA pellet was washed with 75% ethanol. Ethanol was decanted and pellet was dried at room temperature for 10 min. RNA was dissolved in 20–50 μl of Tris-EDTA (Pharmacia Biotech, Piscataway, NJ) and stored at −70°C until used.

**Gels for RNA and DNA.** All solutions for RNA gels and northern blots were made with molecular biology-grade water. Formaldehyde-agarose gels were assembled using 1% agarose (Amresco, Solon, OH), formaldehyde and 1 × MOPS buffer (Sigma). Ethidium bromide RNA loading buffer (5Prime-3Prime, Boulder, CO) was added to each RNA sample and loaded directly onto gel without heating at 65°C. Molecular weight standards (high and low RNA standards, Gibco-BRL) were heated at 65°C for 10 min before loading onto gel. Agarose (1%)-Tris Borate EDTA gels containing ethidium bromide were used for DNA samples.

**Digoxigenin-Labeled Riboprobes.** A plasmid containing tick calreticulin cDNA (p161A) was used to generate an antisense riboprobe. Briefly, the plasmid was cut with EcoRI to linearize the plasmid. Then, digoxigenin-11-UTP (Boehringer-Mannheim, Indianapolis, IN) and Ambion T7 RNA polymerase Megascript kit (Austin, TX) were used to generate the riboprobe. A sense probe was generated by cutting the plasmid with Dra I and using T3 RNA polymerase (Maxiscript, Ambion, Austin, TX).

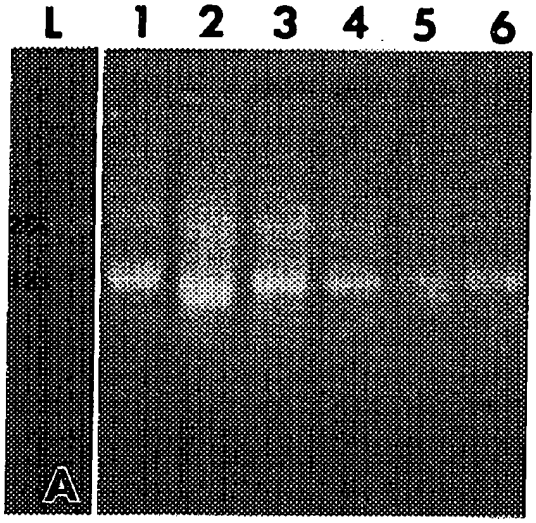
**Northern and Southern Blotting.** Northern transfer was accomplished by capillary blotting to Hybond Plus (Amersham, Arlington Heights, IL) overnight in 20× SSC. DNA gels were denatured, neutralized, and transferred by capillary action in 20× SSC. Blots were UV-linked for 30 s at 1,200 μJ. Prehybridization and hybridization solutions were prepared using the Genius System blocking reagent (Boehringer-Mannheim), 50% formamide, 0.02 g Sarkosyl, and 0.01% SDS. Digoxigenin-labeled riboprobes were added at 25 ng/ml of hybridization solution. Hybridization and washings were carried out at 55°C. Hybridizing bands were detected with antidigoxigenin antibody conjugated to alkaline phosphatase. NBT (nitroblue tetrazolium salt) and X-phosphate (5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt) were used as substrate for alkaline phosphatase.

**Polymerase Chain Reaction and Cloning.** 5′ RACE assay (Gibco-BRL) was performed using calreticulin primers derived from published calreticulin sequences and the tick calreticulin. PCR products were directly cloned into pcrSCRIPT (Stratagene, La Jolla, CA).

**Antibodies.** Two polyclonal antibodies were used for immunoblotting and immunofluorescent assay. The 1st antibody is specific for rabbit skeletal muscle calreticulin was made in a goat and recognizes calreticulin as a polypeptide from 58–60 kDa in most tissues surveyed (Michalak et al. 1992). The 2nd polyclonal antibody (anti-161A) was produced against the expressed tick fusion protein (161A) that was subsequently identified as tick calreticulin (Jaworski et al. 1995).

**Electrophoresis and Western Blotting.** Polyacrylamide-SDS minigels (10%, 0.75 mm) were used for all experiments. All electrophoresis and immunoblotting were performed as described previously (Jaworski et al. 1990). Briefly, flea tissues, including explanted flea salivary glands, were homogenized in 10 mM Tris-HCl (pH 7.0), 20 mM EGTA, and 100 μM phenylmethansulfonyl fluoride (PMSF), briefly centrifuged and supernatant was removed. SDS (4×) Laemmeli loading buffer was added to the supernatant, boiled for 5 min, and loaded onto the gel. Standard immunoblotting procedures were used (Towbin et al. 1979). Primary antibodies were used at 1:100 for anti-161A and 1:200 for anticalreticulin. Secondary antibodies congugated to horseradish peroxidase were directed toward rabbit IgG for anti-161A and toward goat IgG for anticalreticulin and were used at 1:1,000. Antigen–antibody complexes were visualized with TMB (3, 3′ 5, 5′-Tetramethylbenzidine, Kirkegard and Perry, Gaithersburg, MD), peroxidase substrate for all immunoblots.

**Immunofluorescence Antibody Assay.** Adult fleas were pierced with a minutin insect pin and placed in Streck tissue fixative (Streck, Omaha, NE). Tissue was processed, embedded in paraffin, and 4-μm serial sections were cut. Sections were then deparaffinized with xylene, rehydrated, and soaked in PBS for 30 min. Slides were dried before the indirect immunofluorescent antibody assay (IFA). Briefly, primary antibodies (1:200 for anticalreticulin and 1:100 for anti-161A) were diluted in PBS and sections were incubated for 30 min in



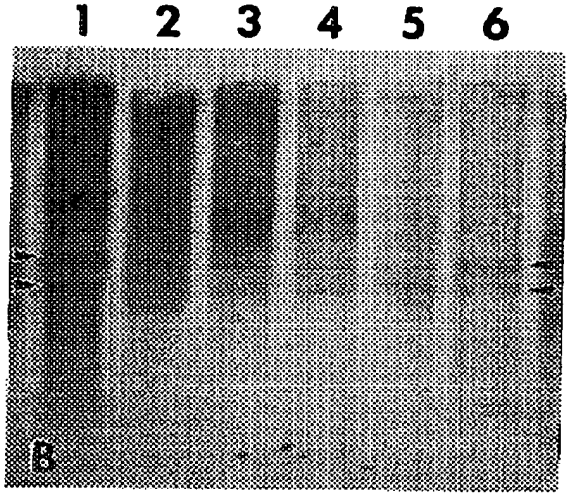




**Fig. 1.** Cat flea RNA gel (panel A) and corresponding Northern Blot (panel B) using antisense riboprobe made from a plasmid containing tick calreticulin (161A). Lanes 1–3 each contain ≈20 μg of total RNA. Lanes 4–6 each contain ≈10 μg of total RNA. Lane L, rat liver RNA used as a marker. Lanes 1 and 6, 14-d fed female. Lanes 2 and 5, unfed male flea. Lanes 3 and 4, fed male. Prehybridization and hybridization at 55°C in 50% formamide. Washes at 55°C. Antidigoxigenin, 1:1,000.

this solution at room temperature. Next, slides were washed 5 times in PBS. Secondary antibodies (antigoat and antirabbit, Kirkegard and Perry) were diluted 1:40 in PBS containing 0.1% Evans blue. Secondary antibody solutions were incubated over slides for 30 min at room temperature. Slides were washed in PBS 5 times and a drop of Vectastain slow-fade (Vector, Burlingame, CA) was applied before the coverslip and viewing with a fluorescent microscope.

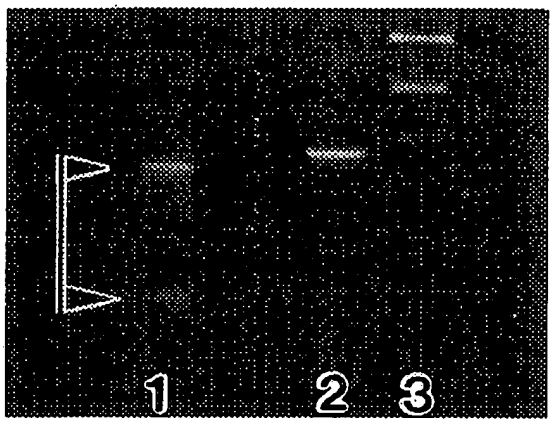



**Fig. 2.** Cat flea PCR products from 5′ RACE. One microgram of 14-d fed female mRNA was used as template to make cDNA. Gene-specific probes (GSP) were as follows: GSP 1-CRT3AS, 5′-ctttgctgcaccacaatgggg-3′; nested GSP for calreticulin, 5′-gcctcggggtccttgatctt-cttgggcg-3′. Lane 1, amplified flea products shown in lane range from ≈650 bp to <400 bp. Lane 2, control 738-bp product. Lane 3, DNA mass ladder (Gibco-BRL); 2,000-, 1,200-, 800-, 400-bp fragments are visible on the gel.

## Results

Gel analysis of total RNA from unfed and fed fleas shows that flea RNA is composed of typical ribosomal RNA subunits, 28S and 18S (Fig. 1A). In agarose gels run for less time, smaller ribosomal RNAs were also observed. RNA gel analysis showed that fed females have at least twice the amount of RNA found in that of unfed females and males. Northern blot analysis revealed that both male and female cat fleas have transcripts for calreticulin at 1.6 and 2.0 kb (Fig. 1B). The sense riboprobe for calreticulin did not hybridize to the flea RNAs. Attempts at amplifying directly from flea DNA have to this point been unsuccessful. In addition, we used a degenerate calreticulin primer designed from known calreticulin sequences and provided by G. Needham (The Ohio State University, Columbus) that was being used to amplify the 5′ end of the tick calreticulin gene to amplify calreticulin from flea mRNA. Using this method, we were able to amplify a portion of the cat flea calreticulin gene using mRNA from fed female cat fleas and gene specific probes to tick calreticulin (Fig. 2). We cloned the flea-specific products and then amplified specific products from our flea calreticulin clones (Fig. 3). Southern blot analysis demonstrated that several of these flea clones do contain inserts for calreticulin (Fig. 3).

Continuously grown embryonic flea cells reacted similarly with anticalreticulin and anti-161A antibodies (compare Fig. 4 B and C). Immunofluorescent antibody assays show a general labeling of flea tissues with calreticulin antibody having several sites of more specific binding. Localization of calreticulin antibody to flea tissues (Fig. 4D) indicates

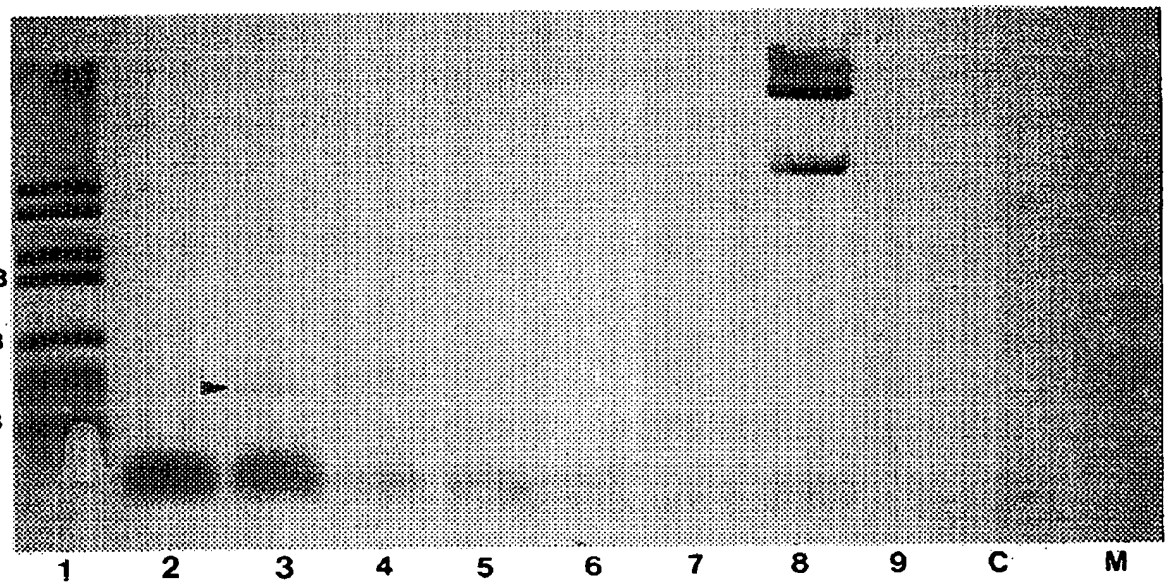



**Fig. 3.** Southern hybridization to riboprobe 161A. Lane 1, digoxigenin-labeled DNA marker VI (Boehringer-Mannheim), molecular weights are indicated on the figure. All products were amplified using T7 and T3 primers in the PCR reaction. Lane 2, pUC plasmid. Lanes 3–7, PCR script containing cat flea insert. Hybridization in lanes 3 and 4 is shown to 450-bp amplification product. Lanes 8–9, probe hybridization to uncut pBluescript containing tick insert calreticulin. Lane C, contamination control. Lane M, DNA mass ladder. Arrows on the figure indicate products that specifically hybridized to riboprobe 161A. The amount of DNA in each lane varied and products were not visible by ethidium bromide staining in every lane. Prehybridization and hybridization at 55°C in 50% formamide. Washes at 55°C. Antidigoxigenin, 1:1,000.

that the midgut reacted, whereas the salivary glands did not crossreact. We also observed specific labeling of calreticulin antibody to the ovaries of fed females (Fig. 4E). Antibody to the tick-secreted protein did not label the ovaries and was only minimally detectable in the midgut of the cat flea. Control tissues showed no labeling of the ovaries or other flea cells (Fig. 4F). Autofluorescence of cuticle-containing structures was observed in all treatments.

By immunoblot, one cat flea polypeptide strongly crossreacts with anticalreticulin antibody, and another reacts weakly (Fig. 5A). We did not detect a protein equivalent to the tick-secreted calreticulin in cat flea tissues (Fig. 5B). When looking at explanted flea salivary glands, only the oriental rat flea glands have clearly detectable crossreactive polypeptides to both anticalreticulin (Fig. 5C) and anti-161A antisera (Fig. 5D). The ovaries are labeled along with the midgut microvilli. IFA of the cat flea salivary glands showed no apparent reaction; however, in immunoblots of cat flea salivary glands a calreticulin antigen is faintly visible. The antibody to 161A did not react with any cat flea antigens by IFA or immunoblotting. With antibody to calreticulin, 2 cat flea proteins were crossreactive. Crossreactive calreticulin and tick-secreted calreticulin antigens were observed in immunoblots of salivary glands of *X. cheopis*. The 2 antibodies crossreact with human calreticulin; however, the flea antigens recognized by these antibodies appear to be different. *X. cheopis* appeared to share epitopes with tick-secreted calreticulin, whereas *C. felis* had no detectable epitopes for this calreticulin.

## Discussion

A flea calreticulin gene was expressed in each of the adult stages studied; however, differential gene expression was not observed. Differences in levels of transcripts could be attributed to increased amounts of RNA in fed adults, and particularly, in females. No induced expression with blood feeding in the adult flea was observed, suggesting that this protein may have a housekeeping role for the flea. The presence of 2 transcripts suggests that 2 isoforms of calreticulin were present in fleas. At least 2 transcripts for calreticulin were found in Drosophila flies and Anopheline mosquitoes by Northern blot analysis (unpublished data). The mRNA for calreticulin in rabbit tissues was a transcript of 1.9 kb and under conditions of high stringency and longer exposure time, a transcript of 3.75 kb was also detected (Fliegel et al. 1989). The identity of this transcript was not clear and the authors suggest that it was either an unspliced mRNA species or an mRNA species encoding a different protein which shares sequence similarity with calreticulin. However, for other calreticulins, isoforms have been identified (Liu et al. 1993, Treves et al. 1992).

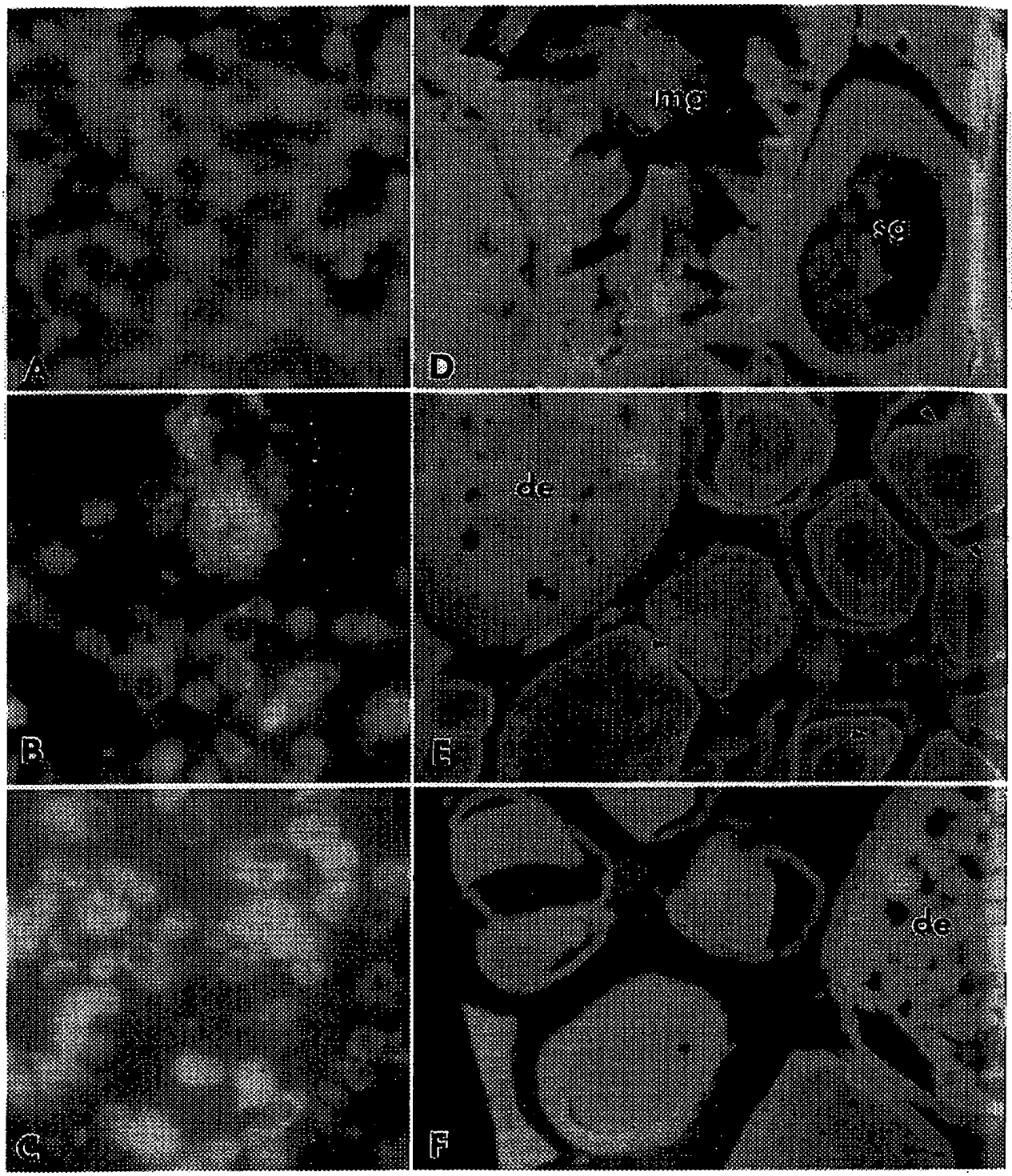



**Fig. 4.** Immunofluorescent antibody staining of cat flea cell line and tissues. Panel A, control embryonic flea cell line, no primary antibody. Panel B, embryonic flea cell line versus anticalreticulin antibody. Panel C, embryonic flea cell line versus anti-161A antibody. Panels D and E, anticalreticulin antibody versus cat flea tissues. (D) Fed female flea showing unlabeled salivary gland (sg) and labeled midgut microvilli (mg). (E) Ovaries of fed female flea. (F) Control tissue, no primary antibody. Arrows on the figure indicate sites of specific labeling of probe or antibody. Developing ovaries (de).

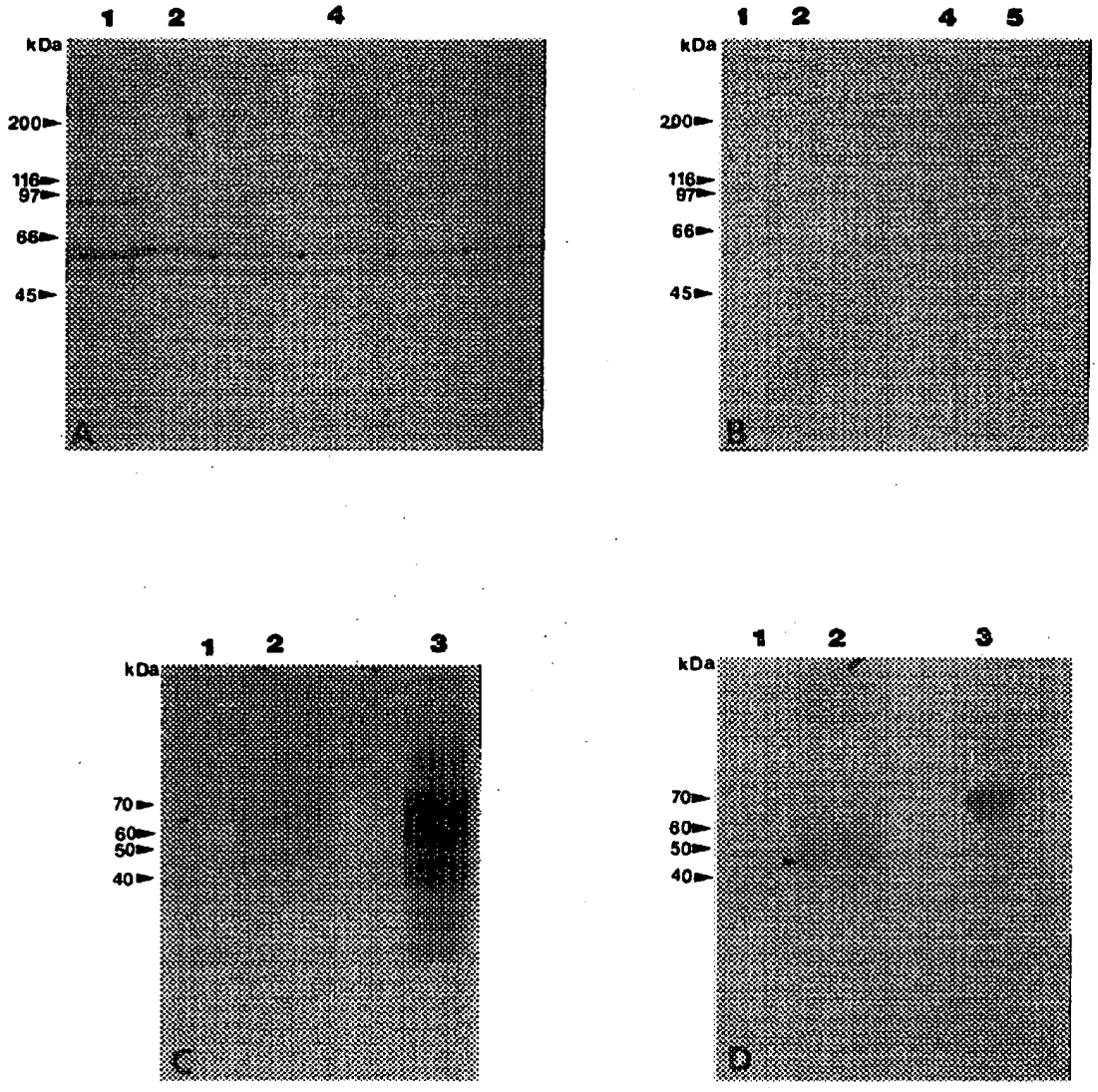



**Fig. 5.** Immunoblots of flea tissues versus anti-161A (tick-secreted calreticulin) and anticalreticulin antibodies. Panel A, Western blot of cat flea proteins versus anticalreticulin antibody. Lanes 1–3, female fleas, 14 d, 1 d, and unfed, respectively. Lanes 4–6, male fleas, 14 d, 1 d, and unfed, respectively. Panel B, Western blot of cat flea proteins versus anti-161A antibody. Lanes 1–3, female fleas, 14 d, 1 d, and unfed, respectively. Lanes 4–6, male fleas, 14 d, 1 d, and unfed, respectively. Panel C, immunoblot of cat flea and rat flea salivary glands versus anticalreticulin antibody. Lane 1, cat flea salivary glands (10 pairs). Arrow depicts barely visible reactivity. Lane 2, rat flea salivary glands (10 pairs). Lane 3, human calreticulin fusion protein. Panel D, immunoblot of cat flea and rat flea salivary glands versus anti-161A. Lane 1, cat flea salivary glands (10 pairs). Lane 2, rat flea salivary glands. Lane 3, human calreticulin fusion protein expressed, but not purified. Positions of molecular weight markers indicated on the figure.

Calreticulin shown in the flea midgut by IFA suggests an alternative source of the protein in flea secretion facilitated by the flea habit of regurgitating into its host during the feeding process. The presence of calreticulin in the ovaries is interesting and requires further investigation. Calreticulins are generally increased under conditions of cell proliferation and growth (Michalak 1992). It is, therefore, possible that the calreticulin found in the ovaries represents the upregulation of development in this tissue following a bloodmeal. For another invertebrate parasite, *Schistosoma mansoni* (Sambon), calreticulin in adult female worms was localized to vitelline follicules, the ephithelia of the digestive ducts, and the periphery of mature ova (Khalife et al. 1993a).

A KDEL anchoring amino acid sequence is associated with the calreticulins that are retained in the endoplamic reticulum (Michalak et al. 1992). Three parasite calreticulins have been identified and these contain variant KDEL (lys, asp, glu, leu) sequence [HEEL-his, glu,glu,leu; tick calreticulin (Jaworski et al. 1995, HDEL-his, asp, glu, leu; *Schistosoma mansoni* calreticulin (Khalife et al.

1993b)] or no sequence [*Onchocerca volvulus* (Railli t & Henry) (Unnasch et al. 1988)]. Calreticulin in ixodid tick saliva is the strongest evidence suggesting that this protein can be secreted. The missing KDEL anchor and the unusual cellular locations of these calreticulins suggest that these calreticulins also have extracellular functions. It will be interesting to compare carboxy terminus of flea calreticulin with these variant calreticulins and to determine if flea calreticulin is a candidate for extracellular function as a component of flea saliva.

Here, we have observed 2 flea species belonging to 2 genera that vary in regard to having an equivalent protein to the tick-secreted calreticulin. Recent investigations show remarkable differences in the presence of a constitutive calreticulin and the tick-secreted calreticulin in vector arthropods (unpublished data). Our research shows that antibody to the tick-secreted calreticulin recognizes different polypeptides in the arthropods tested, whereas all arthropods studied have a constitutive calreticulin. Studies on arthropod apyrases seem to suggest that vector arthropods often recruit housekeeping proteins for use in blood feeding and that the mechanisms to achieving hematophagy through adenosine diphosphate (ADP)-degrading enzymes appear to be independent (Champagne et al. 1995). At this point, we can only suggest roles for calreticulin in the flea and in flea blood feeding. Some ADP-degrading enzymes like apyrase require calcium as a cofactor (Ribeiro et al. 1990). As a calcium-binding protein, calreticulin could serve to bind calcium in the feeding lesion, and thereby modulate apyrase activity. Additionally, in vitro, calreticulin interacts with endothelial cells and causes a dose-dependent release of nitric oxide; and in a canine thrombosis model, intracoronary infusion of calreticulin prevented occlusion of the coronary artery (Kuwabara et al. 1995). Calreticulin appears to prevent thrombosis by binding to endothelial cells and releasing nitric oxide, thereby inhibiting clot formation (Kuwabara et al. 1995).

Calreticulin may influence the transmission of pathogens by way of the bloodmeal and this possibility is also being studied. It has already been suggested that calreticulin bound by antibody in the host can dramatically change the feeding site (Jaworski et al. 1995). Such changes are likely to influence the transmission of pathogens. Saliva-activated transmission (SAT) has been demonstrated in some arthropods (ticks) and tick-borne encephalitis virus (TBE) (Labuda et al. 1993); and with sandflies and *Leishmania* (Titus and Ribeiro 1988). With TBE, SAT induces changes in the feeding lesion that are used by the virus (Jones et al. 1992). We suspect that calreticulin may have a role in host immunomodulation and saliva-activated transmission.

Further studies are in progress to complete the sequencing of the cat flea PCR product for comparison to tick-secreted calreticulin. Studies of other blood-feeding arthropods at the protein and gene level are also being done. We are particularly interested in comparing the secretions of these arthropods. We hope to further define the expression of calreticulin in fleas, and in general, blood-feeding arthropods, with respect to its role in feeding and pathogen transmission.

## Acknowledgments

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